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Effects of growth temperature and nitrogen nutrition on expression of C₃–C₄ intermediate traits in *Chenopodium album*

Jemin Oono[1] · Yuto Hatakeyama[1] · Takayuki Yabiku[1] · Osamu Ueno[2]

Abstract

Proto-Kranz plants represent an initial phase in the evolution from C_3 to C_3–C_4 intermediate to C_4 plants. The ecological and adaptive aspects of C_3–C_4 plants would provide an important clue to understand the evolution of C_3–C_4 plants. We investigated whether growth temperature and nitrogen (N) nutrition influence the expression of C_3–C_4 traits in *Chenopodium album* (proto-Kranz) in comparison with *Chenopodium quinoa* (C_3). Plants were grown during 5 weeks at 20 or 30 °C under standard or low N supply levels (referred to as 20SN, 20LN, 30SN, and 30LN). Net photosynthetic rate and leaf N content were higher in 20SN and 30SN plants than in 20LN and 30LN plants of *C. album* but did not differ among growth conditions in *C. quinoa*. The CO_2 compensation point (Γ) of *C. album* was lowest in 30LN plants (36 μmol mol^{-1}), highest in 20SN plants (51 μmol mol^{-1}), and intermediate in 20LN and 30SN plants, whereas Γ of *C. quinoa* did not differ among the growth conditions (51–52 μmol mol^{-1}). The anatomical structure of leaves was not considerably affected by growth conditions in either species. However, ultrastructural observations in *C. album* showed that the number of mitochondria per mesophyll or bundle sheath (BS) cell was lower in 20LN and 30LN plants than in 20SN and 30SN plants. Immunohistochemical observations revealed that lower accumulation level of P-protein of glycine decarboxylase (GDC-P) in mesophyll mitochondria than in BS mitochondria is the major factor causing the decrease in Γ values in *C. album* plants grown under low N supply and high temperature. These results suggest that high growth temperature and low N supply lead to the expression of C_3–C_4 traits (the reduction of Γ) in the proto-Kranz plants of *C. album* through the regulation of GDC-P expression.

Keywords C_3–C_4 intermediate photosynthesis · *Chenopodium album* · CO_2 compensation point · Glycine decarboxylase · Growth temperature · Nitrogen nutrition

Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the key enzyme in CO_2 fixation in plants. The product of the oxygenase reaction, 2-phosphoglycolate, is recycled in a process called photorespiration (Bauwe 2011). The glycine decarboxylase (GDC) complex in mitochondria decarboxylates the intermediate metabolite of the photorespiratory (glycolate) cycle, glycine, resulting in the loss of previously fixed CO_2 (Schulze et al. 2016). In C_3 plants, a quarter of

fixed CO_2 is lost through photorespiration under the present atmospheric conditions (Sage et al. 2012). In C_4 plants, a CO_2-concentrating mechanism (C_4 cycle) suppresses the oxygenase function of Rubisco and therefore photorespiration; this cycle relies on cooperation between mesophyll (M) and bundle sheath (BS) cells (Hatch 1987; Leegood 2013; Schlüter and Weber 2020).

C_4 plants are considered to have evolved from C_3 plants mainly in response to a reduced concentration of atmospheric CO_2 (Ehleringer and Monson 1993; Sage et al. 2018). In general, C_4 plants prefer high-light, hot and dry environments, in which photorespiration is accelerated. Water use efficiency (WUE) is higher in C_4 plants than in C_3 plants, because C_4 plants can maintain higher photosynthetic rates under lower stomatal conductance (Ghannoum et al. 2011). Nitrogen is an essential nutrient required for photosynthesis and growth. Nitrogen (N) use efficiency is also higher in C_4 plants than in C_3 plants, because less N is allocated

✉ Osamu Ueno
uenoos@agr.kyushu-u.ac.jp

1 Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Motooka 744, Nishi-ku, Fukuoka 819-0395, Japan

2 Faculty of Agriculture, Kyushu University, Motooka 744, Nishi-ku, Fukuoka 819-0395, Japan

to Rubisco and more to other photosynthetic enzymes and thylakoid components (Brown 1978; Ghannoum et al. 2011).

C_4 plants have been hypothesized to have evolved stepwise via intermediate stages called C_3–C_4 intermediate plants or C_3–C_4 plants (Edwards and Ku 1987; Sage et al. 2012; Schlüter and Weber 2020). Their leaves have Kranz-like anatomy, and the BS cells contain abundant chloroplasts and mitochondria; the CO_2 compensation point (Γ), an index of photorespiration rate, is intermediate between those of C_3 and C_4 plants. The reduced photorespiration is achieved by the glycine shuttle (C_2 cycle) operating between the M and BS cells, which recycles photorespired CO_2 (Monson and Rawsthorne 2000; Rawsthorne et al. 1988; Sage et al. 2012). In C_3–C_4 plants, glycine generated in M cells is transported to BS mitochondria for decarboxylation, because M mitochondria lack GDC activity (Rawsthorne et al. 1988). In the BS cells of most C_3–C_4 plants, mitochondria lie between centripetally located chloroplasts and inner tangential walls. This positioning of organelles suppresses the loss of photorespired CO_2 from BS cells, because photorespired CO_2 from mitochondria is captured by the chloroplasts. Type I C_3–C_4 plants reduce photorespiration by the glycine shuttle, whereas type II C_3–C_4 plants have a weak C_4 cycle together with the glycine shuttle (Monson et al. 1986). C_4 plants are thought to have evolved from C_3 plants via proto-Kranz, type I C_3–C_4, type II C_3–C_4, and C_4-like plants (Edwards and Ku 1987; Sage et al. 2012; Schlüter and Weber 2020; Tashima et al. 2021). In proto-Kranz plants, (i) chloroplasts and mitochondria are arranged centripetally in BS cells, but their numbers are lower than in those in C_3–C_4 plants, and (ii) GDC is expressed in all mitochondria of M and BS cells (Muhaidat et al. 2011; Sage et al. 2013; Voznesenskaya et al. 2013; Yorimitsu et al. 2019).

Knowledge of the ecological and adaptive aspects of C_3–C_4 plants would provide an important clue to the evolution of C_4 plants. In general, C_3–C_4 plants grow in warm or hot regions, reflecting their photosynthetic advantages at high leaf temperatures (Lundgren and Christin 2017; Monson and Jaeger 1991; Sage et al. 2018; Schuster and Monson 1990; Sudderth et al. 2009). However, it is difficult to define a universal niche of C_3–C_4 plants (Lundgren and Christin 2017). Some studies have investigated the effects of growth environments—N supply (Bolton and Brown 1980), CO_2 and O_2 concentrations (Byrd and Brown 1989; Pinto et al. 2011; Vogan and Sage 2012), and temperature (Hereford 2017; Pinto et al. 2011)—on photosynthetic traits of C_3–C_4 plants. However, it is not easy to determine which environmental factors are involved in the development of C_3–C_4 traits. Intraspecific variation of photosynthetic traits in C_3–C_4 species has also been investigated. Sayre and Kennedy (1977) found that in *Mollugo verticillata* (type I C_3–C_4), Γ values differ among populations, and Hereford (2017) reported that this variation is temperature dependent.

Teese (1995) reported little differentiation of Γ among populations of *Flaveria linearis* (type I C_3–C_4), but high temperature appears to reduce photorespiration. Further studies will be required to reveal the effects of the environment on the development of C_3–C_4 traits in various species.

Chenopodium album L. (Chenopodiaceae) is a common weed with worldwide distribution. Although *C. album* was previously described as a C_3 species, our recent study has revealed that *C. album* plants have either proto-Kranz or type I C_3–C_4 traits, probably depending on the locality (Yorimitsu et al. 2019). The *C. album* complex is a taxonomically riddle group (Ohri 2015) and includes individuals with different chromosome numbers (Tanaka and Tanaka 1980). It remains to be verified whether intraspecific variation in photosynthesis occurs within it. However, the species may be in the process of evolutionary transition from proto-Kranz to type I C_3–C_4 stage (Yorimitsu et al. 2019). Thus, one could expect plasticity in the expression of photosynthetic traits of *C. album* in response to environmental changes.

Although C_4 plants have higher N use efficiency than C_3 plants, no significant difference has been found between C_3 and type I C_3–C_4 *Flaveria* species (Monson 1989; Vogan and Sage 2011). A constraint-based modeling study has suggested that N limitation may have facilitated C_4 evolution (Blätke and Bräutigam 2019). On the other hand, in the photorespiratory cycle, NH_3 is released in glycine decarboxylation by GDC in mitochondria (Keys and Leegood 2002; Schulze et al. 2016). In C_3–C_4 plants, this release must occur in BS mitochondria (Monson and Rawsthorne 2000; Sage et al. 2018) and would create an imbalance in N metabolites between BS and M cells (Monson and Rawsthorne 2000). The weak C_4 cycle activity in C_3–C_4 species probably serves to rebalance N metabolites (Mallmann et al. 2014). Close association of mitochondria and chloroplasts in BS cells may also facilitate the capture of photorespiratory NH_3, as in the case of CO_2. However, it is fully unknown whether N nutrition level influences the expression of C_3–C_4 traits.

In this study, we investigated whether the expression of C_3–C_4 traits in the proto-Kranz plants of *C. album* is influenced by growth temperature and N nutrition level. We also examined the C_3 species *Chenopodium quinoa* Willd. as a control. The results of this study suggest that high growth temperature and low N supply promote the expression of C_3–C_4 traits in the proto-Kranz plants of *C. album*.

Materials and methods

Plant materials and growth conditions

Seeds of *C. album* were collected in upland fields in Tsukuba, Ibaraki, Japan. Seeds of *C. quinoa* (cv. Hokukei CG1) were provided by the National Agriculture and Food

Research Organization (NARO) Genebank, Tsukuba. We have previously classified them into the proto-Kranz and C_3 types, respectively (Yorimitsu et al. 2019). The seeds were germinated and the seedlings were grown for 2 weeks in perforated multiwell nursery boxes set in a greenhouse in the experimental field of Kyushu University, Fukuoka, Japan. Seedlings were then transplanted to 5-L pots (one plant per pot) with sandy loam soil containing standard N (SN, 1.0 g N per pot) or low N (LN, 0.05 g N per pot); N was supplied as ammonium nitrate. The N level in SN was the same as in our previous study on *Chenopodium* species (Yorimitsu et al. 2019). Each pot also contained 1.0 g each of phosphorus (as calcium superphosphate) and potassium (as potassium chloride). All plants were grown in the growth chambers of the Biotron Application Center, Kyushu University, for 5 weeks at 20 or 30 °C under natural sunlight (maximum photosynthetic photon flux density about 1000 µmol m^{-2} s^{-1} at plant height). Hereafter, we refer to them as 20SN, 20LN, 30SN, and 30LN plants. Plants were watered once every 2 or 3 days for the first 2 weeks and then daily. The position of plants in the growth chambers was changed every week to avoid positional effects. Recently expanded mature leaves of 3 or 4 plants per treatment were used for analysis.

Gas exchange measurements

Gas exchange traits in leaves were measured between 08:00 and 12:00 using an LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA). After acclimatization of the plant to chamber conditions of the system to achieve steady-state CO_2 and H_2O fluxes, net photosynthetic rate (A) was measured at a photosynthetic photon flux density of 1000 µmol m^{-2} s^{-1}, a leaf temperature of 30 °C, a relative humidity of 60%, and a CO_2 concentration of 380 µL L^{-1}. These conditions were the same as in Yorimitsu et al. (2019). Light within the chamber was provided by a 6400-02 LED Light Source (Li-Cor Inc.). Measurement of the CO_2 compensation point (Γ) was started at a CO_2 concentration of 380 µL L^{-1}, and then CO_2 concentration was lowered stepwise until A became negative; the CO_2 concentration was then increased stepwise to 380 µL L^{-1}. The Γ values were determined by extrapolating the initial slope of A versus the intercellular CO_2 concentration (C_i) through the x-axis, where $A = 0$ (Yorimitsu et al. 2019). Carboxylation efficiency was calculated from the initial slope. Photosynthetic WUE was calculated as PWUE = $A \div$ transpiration rate (T_r).

Leaf mass per area, N content, and photosynthetic N use efficiency

Leaves used for the gas exchange measurements were also used to determine leaf mass per area (LMA). Leaf samples

(2.2 cm^2) were air dried for 1 day at 70 °C and weighed. LMA was calculated as dry weight $\div$ leaf area. Several dried leaves, including those used for LMA determination, were ground to a fine powder with a pestle in a mortar, and the N content of each sample (0.3 g of powder) was determined by a micro-Kjeldahl procedure (Tsutsumi et al. 2017). Photosynthetic N use efficiency (PNUE) was calculated as $A \div$ leaf N content.

Anatomy

Leaves of *C. album* and *C. quinoa* were sampled in the morning of a fine day. Segments excised from the middle between the leaf tip and base (about 1 mm $\times$ 1.5 mm) were fixed in 3% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at room temperature for 1.5 h, washed with phosphate buffer, and post-fixed in 2% (w/v) OsO_4 in phosphate buffer for 2 h. Then they were dehydrated through an acetone series and embedded in Quetol resin (Nisshin EM, Shinjuku, Tokyo, Japan). Semithin Sects (1 µm thick) were cut with a glass knife on an ultramicrotome (Porter-Blum MT-2B, Sorvall Inc., Norwalk, CT, USA), mounted on glass slides, stained with 1% (w/v) toluidine blue O, and observed under a light microscope (Eclipse Ci-L, Nikon Instech Co., Ltd., Tokyo, Japan). For *C. album*, the profile areas of M and BS tissues between adjacent small vascular bundles were measured in ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the M/BS tissue area ratio was calculated. The sizes (profile areas) of 5 M cells and 5 BS cells per plant were also measured, and the M/BS cell size ratio was calculated.

To measure vein density in *C. album* leaves, leaf segments (1.8 mm^2) were fixed in a formalin–acetic acid–alcohol mixture and cleared in 80% (v/v) lactic acid and chloral hydrate–saturated ethanol as described by Tsutsumi et al. (2017). The vein density (vein length per unit leaf area) was measured in ImageJ software.

Quantification of chloroplasts and mitochondria

Chloroplasts and mitochondria were quantified in M and BS cells of *C. album*. Ultrathin sections were cut from the leaf samples embedded in Quetol resin with a diamond knife on the ultramicrotome, picked up on Formvar-coated copper grids, stained with lead citrate, and viewed under a transmission electron microscope (JEM-100CX II K, JEOL Ltd., Tokyo, Japan) at 75 kV. The numbers of chloroplasts and mitochondria per cell were counted in 5 M cells and 5 BS cells per plant. The intracellular positions of these organelles in BS cells were determined following the definition of Hatakeyama and Ueno (2016). We counted chloroplasts and mitochondria in the inner halves (i.e., along the inner tangential wall and the inner half of the radial wall) and in

the outer halves (i.e., along the outer tangential wall and the outer half of the radial wall) of the BS cells. The sizes (profile areas) of 10–15 chloroplasts and 10–15 mitochondria per plant were measured in ImageJ software.

Immunohistochemistry

Segments excised from the middle between leaf tip and base (about 0.5 cm × 1 cm) were fixed in 3% (w/v) paraformaldehyde with 0.2% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer (pH 6.8) at 4 °C for 10 h. The segments were washed with phosphate buffer, dehydrated through an ethanol–tertiary butanol series, and embedded in Paraplast X-TRA (Fisher Scientific Co., Houston, TX, USA) as described by Hatakeyama and Ueno (2016). Sections (10 μm thick) were cut on a rotary microtome (PR-50, Yamato Koki Co., Ltd., Saitama, Japan), mounted on glass slides coated with poly-L-lysine (Sigma-Aldrich, Inc., St Louis, MO, USA), and dried overnight at 46 °C. Antiserum for P-protein of GDC (GDC-P) was the same as in our previous study (Yorimitsu et al. 2019). Sections were immunostained for GDC-P as described by Hatakeyama and Ueno (2016), except that the antiserum dilution was 1:1000.

Western blots

Leaves were sampled, frozen immediately in liquid nitrogen, and stored in a deep freezer (−80 °C). Extraction of soluble proteins, SDS-PAGE, and Western blotting were done as described by Ueno (1992). Antisera for Rubisco large subunit (LSU) and GDC-P were the same as in Yorimitsu et al. (2019); the antisera were diluted 1:1000.

Statistical analysis

Data are presented as means of 4 plants ± SE for the gas exchange traits and means of 3 plants ± SE for other physiological traits of *C. album* and *C. quinoa*. Data for the structural traits of *C. album* are presented as means of 3 plants ± SE. The data were analyzed in Statcel 4 software (OMS Publisher, Saitama, Japan). The significance of differences in physiological and structural traits among 20SN, 20LN, 30SN, and 30LN plants in each species was tested by ANOVA, followed by Tukey–Kramer post hoc tests. *P* values less than 0.05 were considered statistically significant.

Results

Gas exchange and other physiological traits

In *C. album*, *A* was lower in 20LN or 30LN plants than in 20SN and 30SN plants, with the lowest value in 20LN

plants (Fig. 1a). There were no differences in T_r among the growth conditions in *C. album*, although T_r tended to be lower in 20LN plants (Fig. 1b). Γ of *C. album* was lowest in 30LN plants (36 μmol mol^{-1}) and highest in 20SN plants (51 μmol mol^{-1}) (Fig. 1c). The carboxylation efficiency of *C. album* was lower in 20LN than in 20SN plants, and in 30LN than in 30SN plants, with the lowest value in 20LN plants (Fig. 1d). In *C. quinoa*, there were no significant differences in *A*, T_r, Γ, or carboxylation efficiency among the growth conditions (Fig. 1a–d). Γ of *C. quinoa* was within a narrow range (51–52 μmol mol^{-1}). In line with N supply level, the leaf N content of *C. album* was higher in 20SN and 30SN plants than in 20LN and 30 LN plants (Fig. 1e). A similar trend was observed in *C. quinoa*, but with no significant differences among the growth conditions (Fig. 1e). On the other hand, LMA of *C. album* was highest in 20LN and lowest in 30SN plants (Fig. 1f). In *C. quinoa*, LMA was higher in 20LN than in 30SN and 30LN plants and intermediate in 20SN plants (Fig. 1f). In both species, there were no significant differences in PNUE (Fig. 1g) or PWUE (Fig. 1h).

Leaf anatomy

In the leaves of *C. album* and *C. quinoa*, the M was differentiated into palisade and spongy tissues (Figs. 2, 3). The BS cells of *C. album* contained many centripetally located chloroplasts in all growth conditions (Fig. 2a–d), which are typical of proto-Kranz type. The M and BS cells of *C. album* accumulated abundant starch grains in all growth conditions (Fig. 2a–d, S1, S2). In all growth conditions of *C. quinoa,* the BS cells contained fewer chloroplasts than those of *C. album*, and much fewer of them were located centripetally (Fig. 3a–d), which are typical of non-Kranz type.

Quantitative analysis of structural traits in *C. album* leaves

Quantitative responses of anatomical traits and organelles in *C. album* leaves were analyzed to understand the structural basis for the Γ dependence on growth conditions (Table 1). Vein density was lower in 30LN plants than in 30SN plants, and intermediate in 20SN and 20LN plants (Table 1). There were no significant differences in tissue areas of M and BS or in M/BS tissue area ratios among the growth conditions (Table 1). Likewise, there were no significant differences in M and BS cell sizes or M/BS cell size ratios (Table 1).

Electron microscopic observations confirmed that the BS cells of *C. album* leaves had the structural traits of the proto-Kranz type in all four growth conditions: considerable numbers of chloroplasts and mitochondria were distributed centripetally, and the mitochondria tended to be located between the chloroplasts and the inner tangential walls (Fig. S1). Although the chloroplasts of both BS and
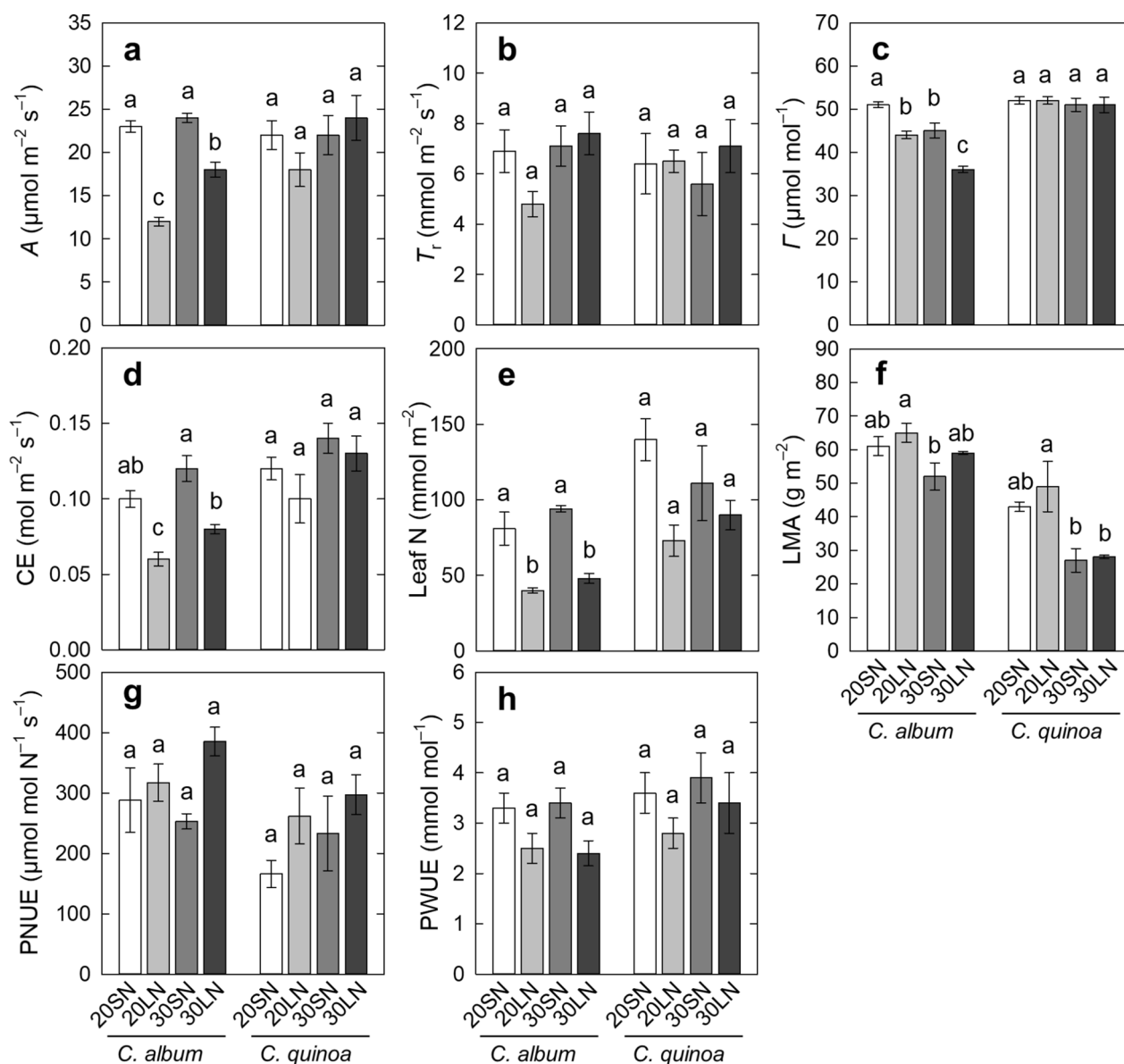
Fig. 1 Photosynthetic and other physiological traits in leaves of *Chenopodium album* and *Chenopodium quinoa* plants grown under different temperatures and N supply levels. **a** Net photosynthetic rate (A). **b** Transpiration rate (T_r). **c** CO_2 compensation point (Γ). **d** Carboxylation efficiency (CE). **e** Leaf N content. **f** Leaf mass per area (LMA). **g** Photosynthetic N use efficiency (PNUE). **h** Photosynthetic water use efficiency (PWUE). *20SN*, 20 °C and standard N level; *20LN*, 20 °C and low N level; *30SN*, 30 °C and standard N level; *30LN*, 30 °C and low N level. Data for gas exchange traits are means ± SE of 4 plants. Data for leaf N content, LMA, and PNUE are means ± SE of 3 plants. Different letters indicate significant difference at $P < 0.05$ in each species

M cells accumulated abundant starch grains, there were more starch grains in 20LN and 30LN plants (Fig. S1b, d, S2b, d) than in 20SN and 30SN plants (Fig. S1a, c, S2a, c). Chloroplast size and number per cell and the distribution of chloroplasts to the inner half of BS cells did not differ significantly among the growth conditions (Table 1). The size of M mitochondria was largest in 30SN and smallest in 20SN, whereas that of BS mitochondria did not differ

significantly among the growth conditions (Table 1). The number of mitochondria per M cell was lower in 20LN and 30LN plants than in 20SN and 30SN plants (Table 1). The number of mitochondria per BS cell showed a similar trend, but only the difference between 20LN and 30SN plants was significant (Table 1). The distribution of mitochondria to the inner half of BS cells was highest in 30SN plants and lowest in 20LN plants (Table 1).
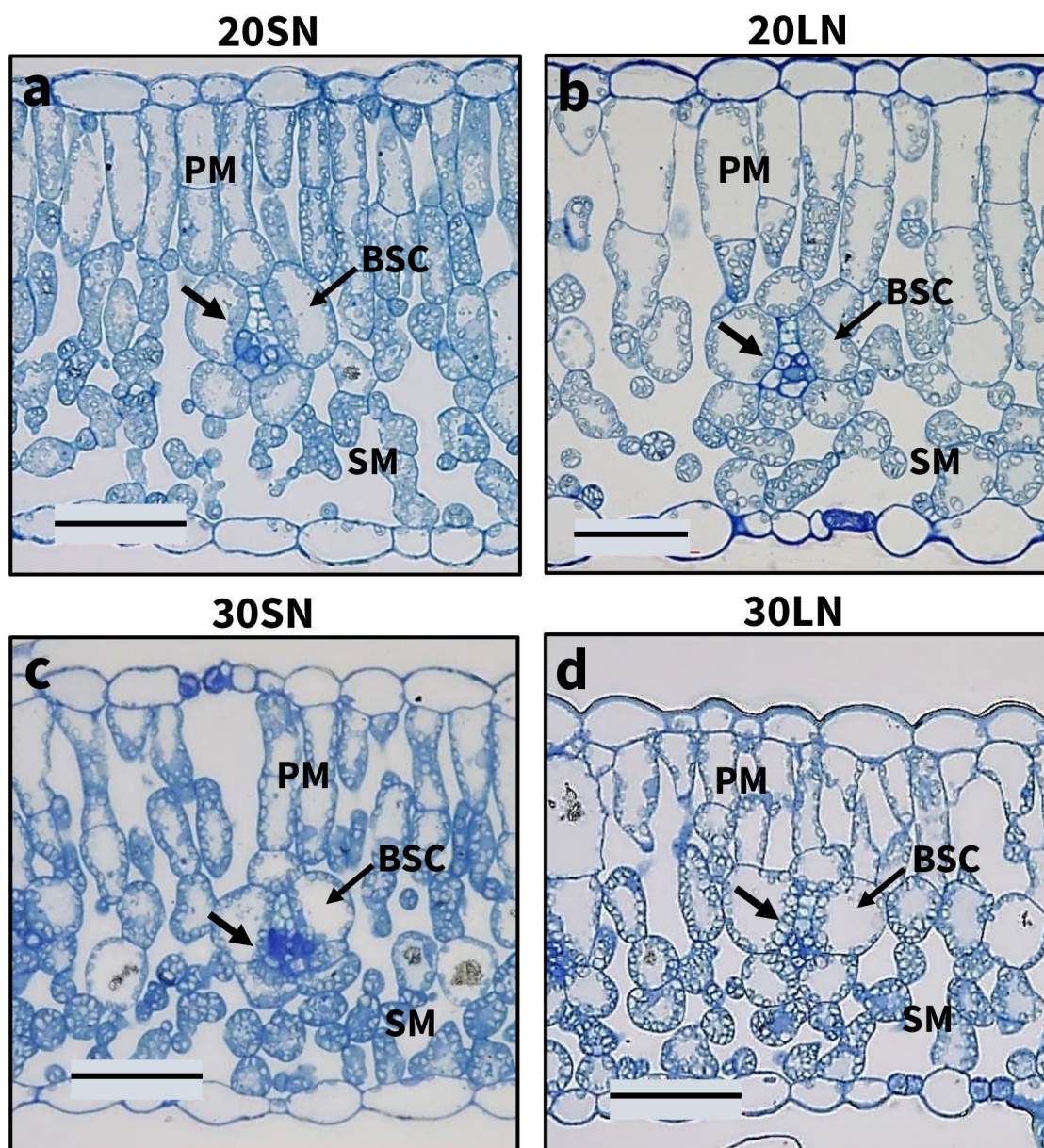
Fig. 2 Leaf anatomy of *C. album* plants grown under different temperatures and N supply levels. Unlabeled arrows indicate centripetally located chloroplasts. *BSC* bundle sheath cell, *PM* palisade mesophyll, *SM* spongy mesophyll. Bars = 50 µm

Immunohistochemical localization of GDC-P

In 20SN and 30SN plants of *C. album*, immunostaining revealed the presence of GDC-P in both M and BS cells, but the BS cells were more strongly stained and formed brown rings, indicating GDC-P accumulation around vascular tissues (Fig. 4a, c). In 20LN and 30LN plants, the overall staining density for GDC-P was weaker (Fig. 4b, d) than in 20SN and 30SN plants (Fig. 4a, c), and was almost undetectable in M cells (Fig. 4b, d). Under the same N level, there was no clear difference in staining between growth temperatures, but staining tended to be stronger in BS cells than in M

cells at 30 °C than at 20 °C (Fig. 4a *vs*. c; Fig. 4b *vs*. d). In *C. quinoa*, GDC-P was present mainly in M cells in all four conditions (Fig. 4e–h). Although BS cells were also weakly stained, the brown rings found in *C. album* were not observed. In *C. quinoa*, the staining density was also somewhat weaker in 20LN and 30 LN plants (Fig. 4f, h) than in 20SN and 30SN plants (Fig. 4e, g).

Western blot analysis of GDC-P and Rubisco LSU

In *C. album* and *C. quinoa*, no large difference was detected in the GDC-P band among the four growth conditions
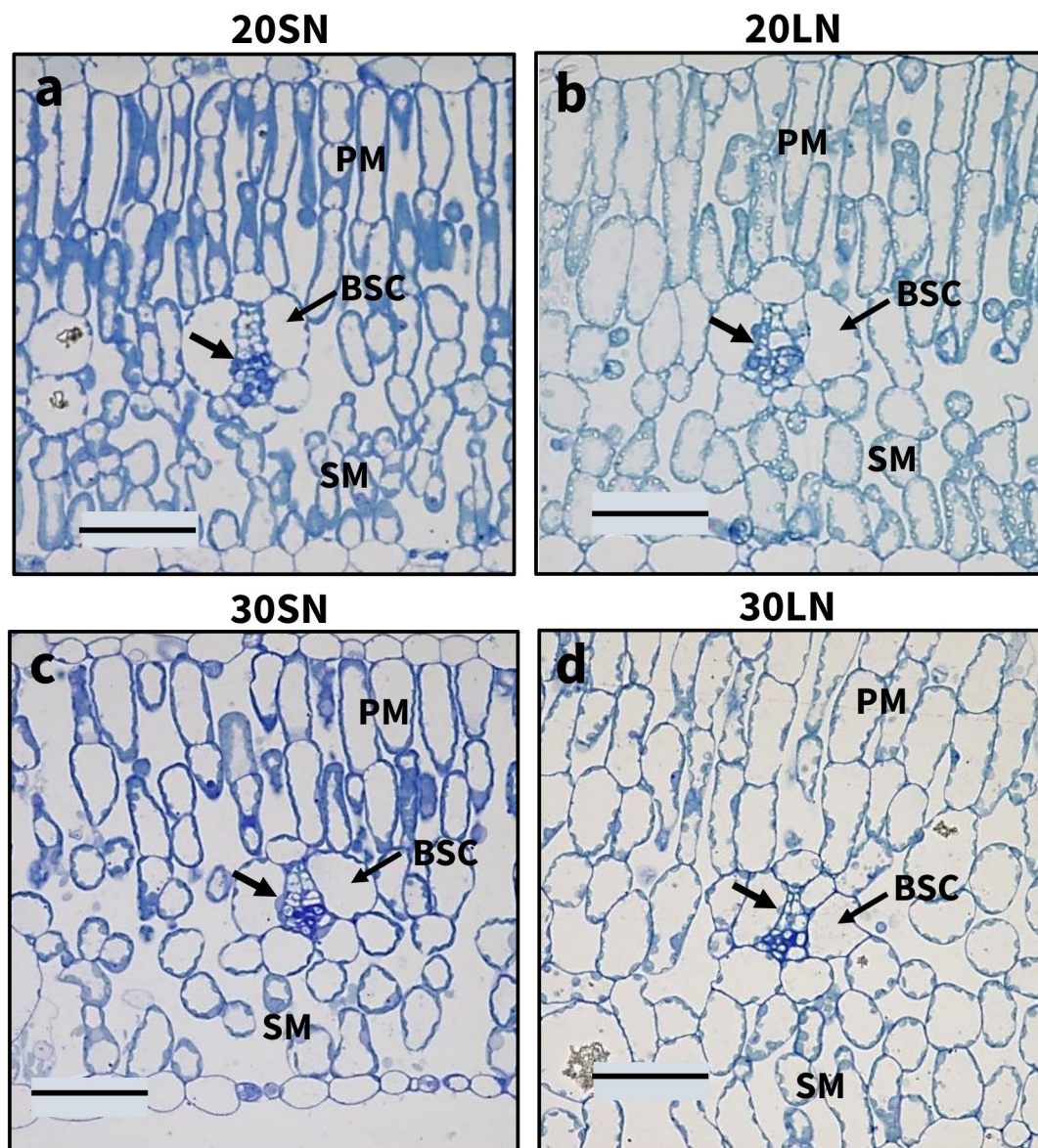
20SN

20LN

30SN

30LN

Fig. 3 Leaf anatomy of *C. quinoa* plants grown under different temperatures and N supply levels. Unlabeled arrows indicate centripetally located chloroplasts. *BSC* bundle sheath cell, *PM* palisade mesophyll, *SM* spongy mesophyll. Bars = 50 μm

(Fig. 5). Likewise, Rubisco LSU was present in all conditions of both species with no distinct differences (Fig. 5).

Discussion

Our study found that the Γ value in *C. album* was lowest under high temperature and low N supply (Fig. 1c). The range of Γ values (36–51 μmol mol^{-1}) found in this study overlapped with those in the proto-Kranz types of *C. album* (38–48 μmol mol^{-1}) and other proto-Kranz species of *Chenopodium* (35–51 μmol mol^{-1}), but was higher than those in type I C$_3$–C$_4$ *Chenopodium* species (20–26 μmol mol^{-1})

(Yorimitsu et al. 2019). These data suggest that apparent photorespiration in *C. album* was reduced at high temperature and low N level, but remained within the range of the proto-Kranz type. It was more strongly reduced by the combination of both factors than by each factor alone. In contrast, growth conditions had no effect on Γ in the C$_3$ control *C. quinoa* plants (51–52 μmol mol^{-1}; Fig. 1c). As expected, A (Fig. 1a) and leaf N content (Fig. 1e) of *C. album* decreased at low N supply. A strong positive correlation between these parameters is well known (Ghannoum et al. 2011; Makino and Ueno 2018). In C$_3$ plants, leaf N content generally correlates positively with LMA (Ghannoum et al. 2011), but no such relationship was observed

Table 1 Comparison of quantitative traits of tissues, cells, and organelles in leaves of *Chenopodium album* plants grown under different temperatures and soil N levels

Trait	Tissue or cell type	20SN[a]		20LN		30SN		30LN	
Vein density (mm mm^{-2})		4.7 ± 0.2	ab	4.9 ± 0.4	ab	5.4 ± 0.1	a	4.1 ± 0.2	b
Tissue area (mm^2)	M[c] tissue	0.027 ± 0.003	a	0.034 ± 0.012	a	0.026 ± 0.005	a	0.043 ± 0.04	a
	BS tissue	0.0053 ± 0.0001	a	0.0056 ± 0.0012	a	0.0047 ± 0.0006	a	0.0067 ± 0.0001	a
M/BS tissue area ratio		5.1 ± 0.4	a	5.9 ± 0.8	a	5.3 ± 0.3	a	6.4 ± 0.5	a
Cell size (μm^2)	M cells	544 ± 40	a	602 ± 106	a	464 ± 39	a	545 ± 72	a
	BS cells	495 ± 32	a	488 ± 55	a	475 ± 27	a	577 ± 55	a
M/BS cell size ratio		1.11 ± 0.12	a	1.22 ± 0.10	a	0.97 ± 0.05	a	0.94 ± 0.07	a
Chloroplast size (μm^2)	M cells	7.6 ± 0.5	a	5.3 ± 0.8	a	8.1 ± 1.0	a	6.1 ± 0.6	a
	BS cells	7.0 ± 0.5	a	4.2 ± 0.3	a	7.2 ± 0.8	a	6.7 ± 1.4	a
Chloroplast number per cell	M cells	17.4 ± 0.4	a	16.7 ± 0.8	a	17.2 ± 0.8	a	14.7 ± 0.9	a
	BS cells	15.2 ± 1.2	a	15.1 ± 1.2	a	17.3 ± 0.6	a	14.9 ± 1.8	a
Chloroplasts in the inner half of BS cells (%)		41 ± 1	a	33 ± 2	a	49 ± 3	a	46 ± 8	a
Mitochondrion size (μm^2)	M cells	0.48 ± 0.03	b	0.55 ± 0.03	ab	0.78 ± 0.09	a	0.53 ± 0.07	ab
	BS cells	1.14 ± 0.05	a	1.04 ± 0.21	a	1.33 ± 0.08	a	1.25 ± 0.08	a
Mitochondrion number per cell	M cells	13.0 ± 2.0	a	3.2 ± 0.2	b	8.6 ± 0.5	a	3.9 ± 0.8	b
	BS cells	10.0 ± 0.9	ab	5.1 ± 1.2	b	12.0 ± 1.4	a	6.4 ± 1.7	ab
Mitochondria in the inner half of BS cells (%)		86 ± 1	ab	78 ± 2	ab	91 ± 1	a	85 ± 3	ab

[a]*20SN*, 20 °C, standard N level; *20LN*, 20 °C, low N level; *30SN*, 30 °C, standard N level; *30LN*, 30 °C, low N level

[b]Values are means ± SE of 3 plants per treatment. Different letters indicate significant difference at *P* < 0.05
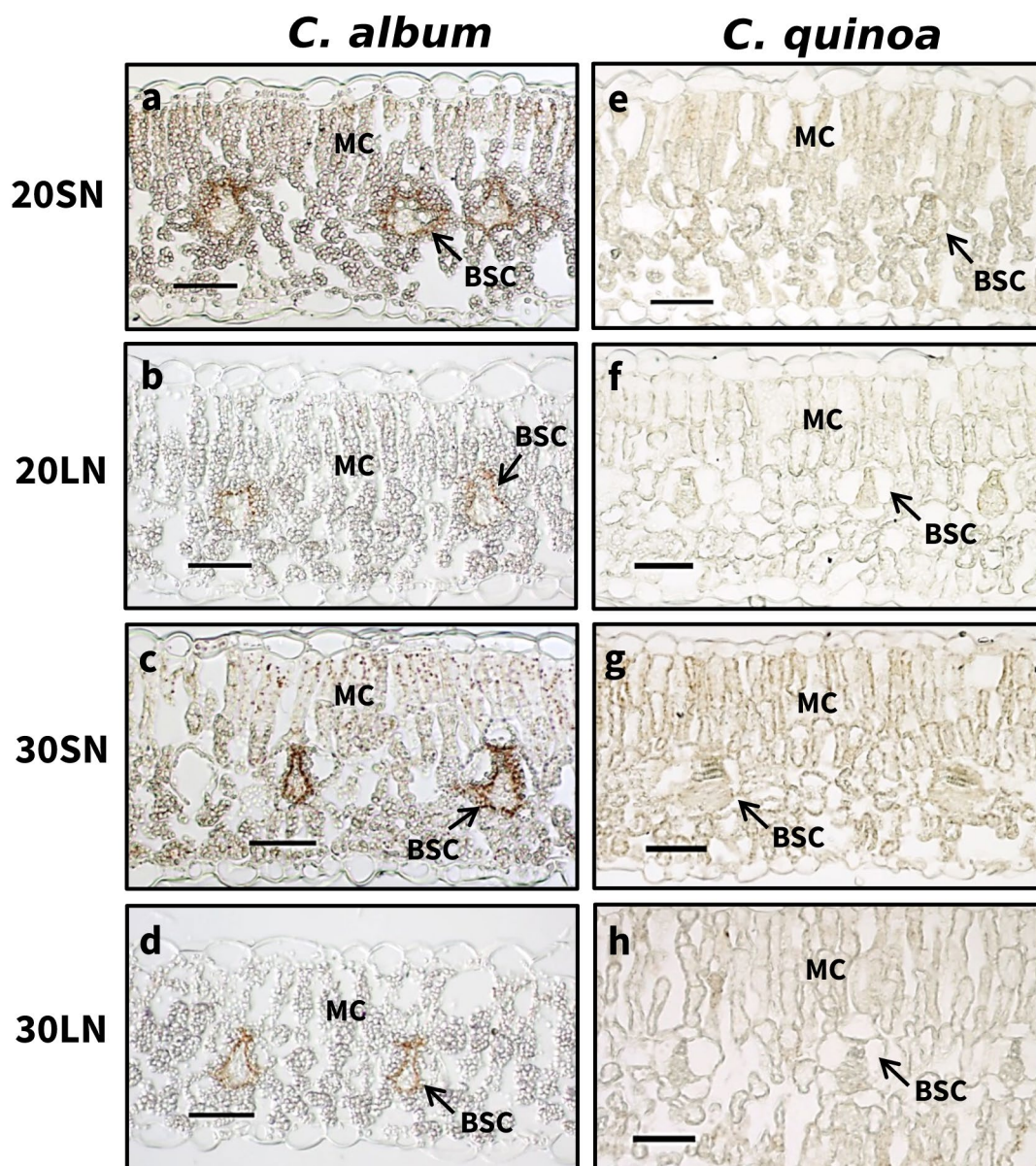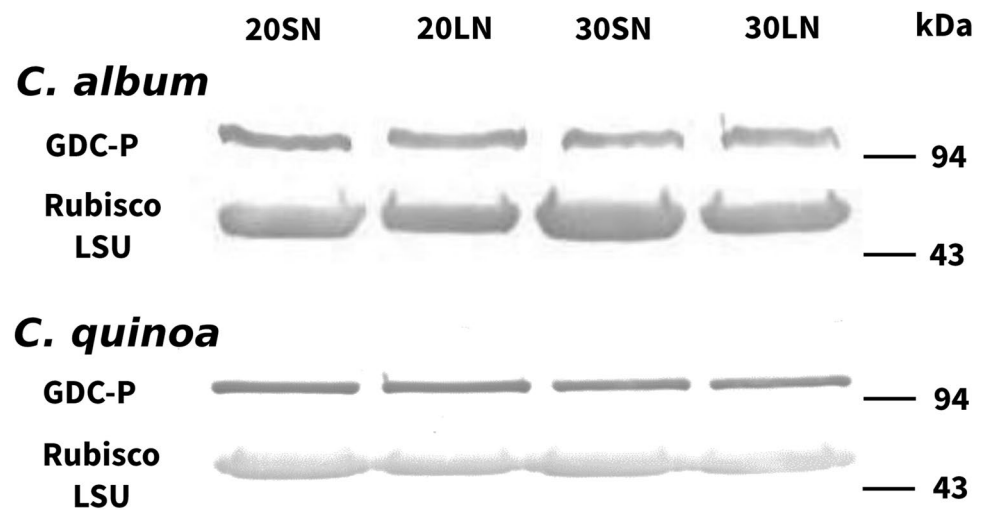
[c]*BS* bundle sheath, *M* mesophyll

Fig. 4 Immunohistochemical localization of glycine decarboxylase P protein in leaves of *C. album* and *C. quinoa* plants grown under different temperatures and N supply levels. *BSC* bundle sheath cell, *MC* mesophyll cell. Bars = 50 μm

in this study in either species (Fig. 1e, f). In *C. album*, no relationship between leaf N content and LMA may be partially explained by the fact that the chloroplasts of 20LN and 30LN plants accumulated more starch grains than those of 20SN and 30SN plants (Figs. S1, S2). Accumulation of starch grains in chloroplasts has also been observed in other plants growing under low N supply (Aviovich and Cresswell 1983; Makino and Ueno 2018).

Light microscopic observations revealed no considerable differences in leaf anatomy of *C. album* or *C. quinoa* among the four growth conditions (Figs. 2, 3). Quantitative analysis of structural traits in *C. album* also showed no significant

differences in M or BS tissue area, tissue area ratio, the sizes of M and BS cells, cell size ratio, chloroplast size, or chloroplast number per cell. On the other hand, the number of mitochondria per cell was generally lower in both M and BS cells of 20LN and 30LN plants than in those of 20SN and 30SN plants, although the size of mitochondria did not differ (Table 1). Overall, it seems that having fewer mitochondria in M and BS cells is a major structural response of *C. album* to low N levels, which may reflect lower physiological activity in LN plants than in SN plants. We expected the distribution ratios of chloroplasts and mitochondria to the inner half of BS cells to increase with a decrease in Γ, because this

organelle positioning is thought to contribute to the low loss of photorespired CO_2 from BS cell mitochondria (Monson and Rawsthorne 2000; Rawsthorne et al. 1988; Sage et al. 2012). These distribution ratios increase with transition from proto-Kranz to C_3–C_4 type in *Chenopodium* (Yorimitsu et al. 2019) and other taxa, such as *Heliotropium* (Boraginaceae; Muhaidat et al. 2011) and *Flaveria* (Asteraceae; Sage et al. 2013). In various hybrids between C_3–C_4 and C_3 species of the Brassicaceae, an increase in the distribution ratios of the organelles to the inner half of BS cells is correlated with the reduction in Γ (Ueno et al. 2003, 2006). In *C. album* in the four growth conditions, however, there were no relationships between the distribution ratios of BS organelles and Γ values (Table 1). These data suggest that factors other than structural traits of BS organelles in leaves were responsible for the reduction in Γ values in *C. album* plants under high temperature and low N supply.

In C_3–C_4 plants, the predominant localization of GDC in BS mitochondria is responsible for the reduction of photorespiration, together with the cell specialization of leaves (Monson and Rawsthorne 2000; Rawsthorne et al. 1988; Sage et al. 2012). Western blot analysis showed that the relative amount of GDC-P in the total soluble protein fraction of *C. album* leaves did not differ greatly among the growth conditions (Fig. 5). On the other hand, the cellular pattern of GDC-P accumulation in *C. album* reflected leaf N contents: the photosynthetic cells were stained more densely in 20SN and 30SN plants than in 20LN and 30LN plants (Fig. 4a–d). In 20SN and 30SN plants, dense staining for GDC-P was observed in both M and BS cells, which is characteristic of the proto-Kranz type (Muhaidat et al. 2011; Sage et al. 2013; Yorimitsu et al. 2019). In 20LN and 30LN plants, the staining in M cells was greatly reduced, whereas that in BS cells remained. This pattern of GDC-P accumulation resembled that found in the C_3–C_4 type (Rawsthorne et al. 1988; Sage et al. 2012). A similar pattern of GDC-P accumulation was

also observed between 20 and 30SN plants and between 20 and 30LN plants, although the difference in accumulation level between the M and BS cells in response to temperature was less distinct than that in response to N level. In *C. quinoa*, GDC-P staining was detected mainly in M cells, and it was weaker in 20LN and 30 LN plants than in 20SN and 30SN plants (Fig. 4e–h) reflecting leaf N content. Taken together, these data suggest that the lower accumulation level of GDC-P in M mitochondria than in BS mitochondria is the major factor causing the decrease in Γ values in *C. album* plants grown under low N supply and high temperature, because main site of release of photorespired CO_2 shifts from M cells to BS cells. Previous studies on C_3–C_4 Brassicaceae species have shown that not a complete lack of GDC-P in M cells, but an increase in GDC-P accumulation in BS cells relative to M cells is required for the decrease in Γ (Ueno et al. 2003, 2006). In the evolution from C_3 to C_3–C_4 plants in *Flaveria*, the expression of two GDC-P genes, *GLDPA* and *GLDPB*, is involved in the cell-specific accumulation of GDC-P (Schulze et al. 2013, 2016). It remains an intriguing issue how the expression of GDC-P genes is regulated in *C. album*.

In C_3 and C_3–C_4 plants, Γ increases with increasing leaf temperature (Brown and Morgan 1980; Fladung and Hesselbach 1989; Vogan and Sage 2012). On the other hand, there are relatively limited data on the effect of growth temperature on Γ. Fladung and Hesselbach (1989) reported that higher levels of light and temperature resulted in a somewhat lower Γ in *Steinchisma hians* (type I C_3–C_4, formerly *Panicum milioides*) but had no effect on Γ in *Panicum bisulcatum* (C_3). These responses of Γ to growth temperature were similar to our results in *Chenopodium* species. It seems that *C. album* plants acclimatize to high growth temperature through the down-regulation of photorespiration, since the acquisition of C_3–C_4 traits is advantageous for the growth of plants in hot and warm regions (Monson and Rawsthorne

2000). Hereford (2017) has reported that when populations of *Mollugo verticillata* (type I C_3–C_4) from a warm and a cool climate were grown in growth chambers at 24 °C or 35 °C, Γ was lower in the warm-climate population at 35 °C and in the cool-climate population at 24 °C. This suggests that the response of photorespiration to temperature differs between populations of the same species and depends on the climate of the growing region. The *C. album* plants examined here originated from a warm temperate region of Japan. It remains unknown whether *C. album* plants from other regions show similar responses to growth temperature.

Bolton and Brown (1980) reported that Γ did not significantly change with increasing N supply in *S. hians*, but decreased somewhat in tall fescue (*Festuca arundinacea*; C_3). In *C. album*, Γ was lower under low N supply (Fig. 1c), but the reduced Γ did not increase PNUE, although PNUE tended to be higher in 30LN plants than in 30SN plants (Fig. 1g). PNUE does not differ significantly between C_3 and type I C_3–C_4 *Flaveria* species (Monson 1989; Vogan and Sage 2011). In leaf mitochondria, equal amounts of CO_2 and NH_3 are released during the conversion of glycine to serine catalyzed by GDC (Keys and Leegood 2002; Schulze et al. 2016). Although NH_3 is rapidly re-assimilated by the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle in chloroplasts, part of it may be emitted from leaves to the atmosphere, resulting in a loss of N from plants (Schjoerring et al. 2000). Thus, the higher accumulation of GDC in BS cells than in M cells may reduce the emission of NH_3 via photorespiration. It will be interesting to know whether the development of C_3–C_4 traits is also associated with plant N economy. In some accessions of *Alloteropsis semialata*, a grass species that includes C_3, C_3–C_4, and C_4 forms, Γ values are lower in moderate nutrient–grown plants than in high nutrient–grown plants (Lundgren et al. 2016). This response is similar to that found in our study, but Γ in these accessions changed from C_3–C_4 to C_4-like values, suggesting that enhanced C_4 cycle activity is probably responsible for the decrease in Γ. Thus, the biochemical and physiological basis for decreased Γ in *A. semialata* would differ from that in *C. album*.

Declarations

References

Aviovich D, Cresswell CF (1983) The effect of nitrogen and phosphorus on starch accumulation and net photosynthesis in two variants of *Panicum maximum* Jacq. Plant Cell Environ 6:657–664. https://doi.org/10.1111/1365-3040.ep11589234

Bauwe H (2011) Photorespiration: the bridge to C_4 photosynthesis. In: Raghavendra AS, Sage RF (eds) C_4 photosynthesis and related CO_2 concentrating mechanisms. Springer, Dordrecht, pp 81–108

Blätke MA, Bräutigam A (2019) Evolution of C_4 photosynthesis predicted by constraint-based modelling. eLife 8:e49305. https://doi.org/10.7554/eLife.49305

Bolton JK, Brown RH (1980) Photosynthesis of grass species differing in CO_2 fixation pathways. V. Response of *Panicum maximum*, *Panicum milioides*, and tall fescue (*Festuca arundinacea*) to nitrogen nutrition. Plant Physiol 66:97–100. https://doi.org/10.1104/pp.66.1.97

Brown RH (1978) A difference in N use efficiency in C_3 and C_4 plants and its implications in adaptation and evolution. Crop Sci 18:93–98. https://doi.org/10.2135/cropsci1978.0011183X001800010025x

Brown RH, Morgan JA (1980) Photosynthesis of grass species differing in CO_2 fixation pathways. VI. Differential effects of temperature and light intensity on photorespiration in C_3, C_4 and intermediate species. Plant Physiol 66:541–544. https://doi.org/10.1104/pp.66.4.541

Byrd GT, Brown RH (1989) Environmental effects on photorespiration of C_3–C_4 species. I. Influence of CO_2 and O_2 during growth on photorespiratory characteristics and leaf anatomy. Plant Physiol 90:1002–1028. https://doi.org/10.1104/pp.90.3.1022

Edwards GE, Ku MSB (1987) The biochemistry of C_3–C_4 photosynthesis. In: Hatch MD, Boardman NK (eds) The biochemistry of plants. Academic Press, New York, pp 275–325

Ehleringer JR, Monson RK (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. Annu Rev Ecol System 24:411–439. http://www.jstor.org/stable/2097185

Fladung M, Hesselbach J (1989) Effects of varying environments on photosynthetic parameters of C_3, C_3–C_4 and C_4 species of *Panicum*. Oecologia 79:168–173. https://doi.org/10.1007/BF00388473

Ghannoum O, Evans JR, von Caemmerer S (2011) Nitrogen and water use efficiency of C_4 plants. In: Raghavendra AS, Sage RF (eds) C_4 photosynthesis and related CO_2 concentrating mechanisms. Springer, Dordrecht, pp 129–146

Hatakeyama Y, Ueno O (2016) Intracellular position of mitochondria and chloroplasts in bundle sheath and mesophyll cells of C_3 grasses in relation to photorespiratory CO_2 loss. Plant Prod Sci 19:540–551. https://doi.org/10.1080/1343943X.2016.1212667

Hatch MD (1987) C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta 895:81–106. https://doi.org/10.1016/S0304-4173(87)80009-5

Hereford J (2017) Genetic divergence for physiological response to temperature between populations of a C_3–C_4 intermediate annual. Int J Plant Sci 178:431–438. https://doi.org/10.1086/691695

Keys AJ, Leegood RC (2002) Photorespiratory carbon and nitrogen cycling: evidence from studies of mutant and transgenic plants.

In: Foyer CH, Noctor G (eds) Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism. Kluwer Academic Publishers, Dordrecht, pp 115–134

Leegood RC (2013) Strategies for engineering C_4 photosynthesis. J Plant Physiol 170:378–388. https://doi.org/10.1016/j.jplph.2012.10.011

Lundgren MR, Christin PA (2017) Despite phylogenetic effects, C_3–C_4 lineages bridge the ecological gap to C_4 photosynthesis. J Exp Bot 68:241–254. https://doi.org/10.1093/jxb/eru186

Lundgren MR, Christin PA, Escobar EG, Ripley BS, Besnard G, Long CM, Hattersley PW, Ellis RP, Leegood RC, Osborne CP (2016) Evolutionary implications of C_3–C_4 intermediates in the grass *Alloteropsis semialata*. Plant Cell Environ 39:1874–1885. https://doi.org/10.1111/pce.12665

Makino Y, Ueno O (2018) Structural and physiological responses of the C_4 grass *Sorghum bicolor* to nitrogen limitation. Plant Prod Sci 21:39–50. https://doi.org/10.1080/1343943X.2018.1432290

Mallmann J, Heckmann D, Brautigam A, Lercher MJ, Weber APM, Westhoff P, Gowik U (2014) The role of photorespiration during the evolution of C_4 photosynthesis in the genus *Flaveria*. eLife 3:e02478. https://doi.org/10.7554/eLife.02478

Monson RK (1989) The relative contributions of reduced photorespiration, and improved water- and nitrogen-use efficiencies, to the advantages of C_3–C_4 intermediate photosynthesis in *Flaveria*. Oecologia 80:215–221. https://doi.org/10.1007/BF00380154

Monson RK, Jaeger CH (1991) Photosynthetic characteristics of C_3–C_4 intermediate *Flaveria floridana* (Asteraceae) in natural habitats: evidence of advantages to C_3–C_4 photosynthesis at high leaf temperatures. Amer J Bot 78:795–800. https://doi.org/10.1002/j.1537-2197.1991.tb14481.x

Monson RK, Rawsthorne S (2000) CO_2 assimilation in C_3–C_4 intermediate plants. In: Leegood RC, Sharkey TD, von Caemmerer S (eds) Photosynthesis: physiology and metabolism. Kluwer Academic Publishers, Dordrecht, pp 533–550

Monson RK, Moore BD, Ku MSB, Edwards GE (1986) Co-function of C_3 and C_4 photosynthetic pathways in C_3, C_4, and C_3–C_4 intermediate *Flaveria* species. Planta 168:493–502. https://doi.org/10.1007/BF00392268

Muhaidat R, Sage TL, Frohlich MW, Dengler NG, Sage RF (2011) Characterization of C_3–C_4 intermediate species in the genus *Heliotropium* L. (Boraginaceae): anatomy, ultrastructure and enzyme activity. Plant Cell Environ 34:1723–1736. https://doi.org/10.1111/j.1365-3040.2011.02367.x

Ohri D (2015) The taxonomic riddle of *Chenopodium album* L. complex (Amaranthaceae). Nucleus 58:131–134. https://doi.org/10.1007/s1323

Pinto H, Tissue DT, Ghannoum O (2011) *Panicum milioides* (C_3–C_4) does not have improved water or nitrogen economies relative to C_3 and C_4 congeners exposed to industrial-age climate change. J Exp Bot 62:3223–3234. https://doi.org/10.1093/jxb/err005

Rawsthorne S, Hylton CM, Smith AM, Woolhouse HW (1988) Photorespiratory metabolism and immunogold localization of photorespiratory enzymes in leaves of C_3 and C_3–C_4 intermediate species of *Moricandia*. Planta 173:298–308. https://doi.org/10.1007/BF00401016

Sage RF, Kocacinar F, Sage TL (2012) Photorespiration and the evolution of C_4 photosynthesis. Annu Rev Plant Biol 63:19–47. https://doi.org/10.1146/annurev-arplant-042811-105511

Sage TL, Busch FA, Johnson DC, Friesen PC, Stinson CR, Stata M, Sultmanis S, Rahman BA, Rawsthorne S, Sage RF (2013) Initial events during the evolution of C_4 photosynthesis in C_3 species of *Flaveria*. Plant Physiol 163:1266–1276. https://doi.org/10.1104/pp.113.221119

Sage RF, Monson RK, Ehleringer JR, Adachi S, Pearcy RW (2018) Some like it hot: the physiological ecology of C_4 plant evolution. Oecologia 187:941–966. https://doi.org/10.1007/s00442-018-4191-6

Sayre RT, Kennedy RA (1977) Ectopic differences in the C_3 and C_4 photosynthetic activity in *Mollugo verticillata*, a C_3–C_4 intermediate. Planta 134:257–262. https://doi.org/10.1007/BF00384190

Schjoerring JK, Husted S, Mäck G, Nielsen KH, Finnemann J, Mattsson M (2000) Physiological regulation of plant-atmosphere ammonia exchange. Plant Soil 221:95–102. https://doi.org/10.1023/A:1004761931558

Schlüter U, Weber APM (2020) Regulation and evolution of C_4 photosynthesis. Annu Rev Plant Biol 71:183–215. https://doi.org/10.1146/annurev-arplant-042916-040915

Schulze S, Mallmann J, Burscheidt J, Koczor M, Streubel M, Bauwe H, Gowik U, Westhoff P (2013) Evolution of C_4 photosynthesis in the genus *Flaveria*: establishment of a photorespiratory CO_2 pump. Plant Cell 25:2522–2535. https://doi.org/10.1105/tpc.113.114520

Schulze S, Westhoff P, Gowik U (2016) Glycine decarboxylase in C_3, C_4 and C_3–C_4 intermediate species. Curr Opin Plant Biol 31:29–35. https://doi.org/10.1016/j.pbi.2016.03.011

Schuster WS, Monson RK (1990) An examination of the advantages of C_3–C_4 intermediate photosynthesis in warm environments. Plant Cell Environ 13:903–912. https://doi.org/10.1111/j.1365-3040.1990.tb01980.x

Sudderth EA, Espinosa-Garcia FJ, Holbrook NM (2009) Geographic distributions and physiological characteristics of co-existing *Flaveria* species in South-central Mexico. Flora 204:89–98. https://doi.org/10.1016/j.flora.2008.01.005

Tanaka R, Tanaka A (1980) Karyomorphological studies on halophytic plants. I Some Taxa of *Chenopodium*. Cytologia 45:257–269. https://doi.org/10.1508/cytologia.45.257

Tashima M, Yabiku T, Ueno O (2021) *Coleataenia prionitis*, a C_4-like species in the Poaceae. Photosynth Res 147:211–227. https://doi.org/10.1007/s11120-020-00808-w

Teese P (1995) Intraspecific variation for CO_2 compensation point and differential growth among variants in a C_3–C_4 intermediate plant. Oecologia 102:371–376. https://doi.org/10.1007/BF00329804

Tsutsumi N, Tohya M, Nakashima T, Ueno O (2017) Variations in structural, biochemical and physiological traits of photosynthesis and resource use efficiency in *Amaranthus* species (NAD-ME-type C_4). Plant Prod Sci 20:300–312. https://doi.org/10.1080/1343943X.2017.1320948

Ueno O (1992) Immunogold localization of photosynthetic enzymes in leaves of *Aristida latifolia*, a unique C_4 grass with a double chlorenchymatous bundle sheath. Physiol Plant 85:189–196. https://doi.org/10.1111/j.1399-3054.1992.tb04722.x

Ueno O, Bang SW, Wada Y, Kondo A, Ishihara K, Kaneko Y, Matsuzawa Y (2003) Structural and biochemical dissection of photorespiration in hybrids differing in genome constitution between *Diplotaxis tenuifolia* (C_3–C_4) and radish (C_3). Plant Physiol 132:1550–1559. https://doi.org/10.1104/pp.103.021329

Ueno O, Wada Y, Wakai M, Bang SW (2006) Evidence from photosynthetic characteristics for the hybrid origin of *Diplotaxis muralis* from a C_3–C_4 intermediate and a C_3 species. Plant Biol 8:253–259. https://doi.org/10.1055/s-2005-873050

Vogan PJ, Sage RF (2011) Water-use efficiency and nitrogen-use efficiency of C_3–C_4 intermediate species of *Flaveria* Juss. (Asteraceae). Plant Cell Environ 34:1415–1430. https://doi.org/10.1111/j.1365-3040.2011.02340.x

Vogan PJ, Sage RF (2012) Effects of low atmospheric CO_2 and elevated temperature during growth on the gas exchange responses of C_3, C_3–C_4 intermediate, and C_4 species from three evolutionary lineages of photosynthesis. Oecologia 169:341–352. https://doi.org/10.1007/s00442-011-2201-z

Voznesenskaya EV, Koteyeva NK, Akhani H, Roalson EH, Edwards GE (2013) Structural and physiological analyses in Salsoleae

(Chenopodiaceae) indicate multiple transitions among C_3, intermediate, and C_4 photosynthesis. J Exp Bot 64:3583–3604. https://doi.org/10.1093/jxb/ert191

Yorimitsu Y, Kadosono A, Hatakeyama Y, Yabiku T, Ueno O (2019) Transition from C_3 to proto-Kranz to C_3–C_4 intermediate type in the genus *Chenopodium* (Chenopodiaceae). J Plant Res 132:839–855. https://doi.org/10.1007/s10265-019-01135-5

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